THE EFFECT OF RIFAMPICIN ON EXPRESSION OF THE TOLL-LIKE RECEPTOR SYSTEM GENES IN THE FOREBRAIN CORTEX OF RATS PRENATALLY EXPOSED TO ALCOHOL

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Ethanol causes long-term changes in the toll-like receptor (TLR) system, promoting activation of neuroinflammation pathways. Alcohol use during pregnancy causes neuroinflammatory processes in the fetus; this can lead to the development of symptoms of fetal alcohol spectrum disorder (FASD). Our study has shown that prenatal alcohol exposure (PAE) induced long-term changes in the TLR system genes (*Tlr3*, *Tlr4*, *Ticam*, *Hmgb1*, cytokine genes) in the forebrain cortex of rat pups. Administration of rifampicin (Rif), which can reduce the level of pro-inflammatory mediators in various pathological conditions of the nervous system, normalized the altered expression level of the studied TLR system genes. This suggests that Rif can prevent the development of persistent neuroinflammatory events in the forebrain cortex of rat pups caused by dysregulation in the TLR system.

Key words: brain; PAE; alcohol; toll-like receptors; neuroinflammation; rifampicin

DOI: 10.18097/PBMC20236904228

INTRODUCTION

Prenatal damage of the central nervous system (CNS) results in the development of neurological and cognitive disorders in children [1–4]. Prenatal alcohol exposure (PAE) causes fetal alcohol spectrum disorders (FASD) [5], in which CNS disorders appear to be the most crucial ones because the fetal CNS is one of the most sensitive systems to maternal alcohol [5–8].

The results of recent studies indicate that inflammatory mediators, including cytokines, are involved in the mechanisms of regulation of higher brain functions [9]. However, there are still not so many publications in the world literature devoted to the study of the state of neuroimmune interactions in the developing brain during PAE, and there is basically no information on the effect of PAE on the innate immune system in the CNS.

The aim of our work was to investigate the effect of PAE on the level of gene expression of the innate immunity system in rats in the forebrain cortex in the neonatal period of development and pharmacological correction of neuroimmune mechanisms in the brain of rat pups with PAE by means of rifampicin (Rif). Previously, Rif showed anti-inflammatory and neuroprotective properties in various models of pathological conditions of the nervous system [10–13].

MATERIALS AND METHODS

Animals

Adult male (250-300 g, n=3) and female (200-250 g, n=6) Wistar rats purchased from the Rappolovo nursery (Russia) were used in the experiment. The animals were kept in separate plastic cages with unlimited access to water and food. Each male was paired with two females. The first day of pregnancy was determined by detection of spermatozoa in females in a vaginal smear. After detection of spermatozoa, female rats were marked, their body weight was determined and they were placed in separate cages, and the countdown of the gestation period was started.

Modeling of Prenatal Alcohol Exposure (PAE)

Pregnant rats were divided into two groups: a group of females exposed to semi-forced alcoholization with 15% ethanol solution as the only source of liquid (n=3) and a control group receiving water (n=3). There were 8-10 pups in each litter; to continue the experiment, 6 pups per litter were left (18 pups with PAE, 18 pups without PAE). Body weight measurements did not reveal any significant differences between groups of animals studied (pregnant rats and offspring).

Administration of Rifampicin (Rif)

Two animals were randomly selected from each litter. During the first 7 days of neonatal development, animals with PAE (PAE + Rif group, n=6) received intraperitoneal (ip) injection of 50 mg/kg rifampicin (Rif; Belmedpreparaty, Belarus). Rif was dissolved in saline. Rat pups of the second (PAE + saline; n=4) and the third (control; n=6) groups received ip injections of an equivalent volume of saline during the first 7 days of neonatal development. On day 8 of postnatal development, the forebrain cortex was sampled and immediately frozen and stored at -78°C.

Real-Time Polymerase Chain Reaction

Total RNA was isolated using the ExtractRNA reagent (Evrogen, Russia) in full accordance with the manufacturer's instructions. Samples were treated with DNase (Promega, USA). Concentrations of the resultant RNA was measured using an Implen NanoPhotometer P330 spectrophotometer (Implen, Germany). The purity of the isolated product was evaluated by the ratio A_{260}/A_{280} (normal ≥1.8). cDNA synthesis was performed by reverse transcription (RT) using the MMLV RT kit (Evrogen) in full accordance with the manufacturer's instructions in the volume of 20 μl. Polymerase chain reaction (PCR) with real-time detection (RT-PCR) was carried out in an Mx3005P amplifier (Stratagene, USA) in 10 μl of the reaction mixture containing SYBR Green MIX (Biolabmix, Russia), a mixture of specific forward and reverse primers (Beagle, Russia), selected using the Primer-BLAST software (Table 1). The data obtained were normalized to the expression level of the *Gapdh* gene and calculated in relative units versus the mRNA content of the studied reference gene (Table 1) using the $2^{\Delta\Delta Ct}$ method [14].

Statistical Data Processing

Statistical processing of results was performed using the program Graph Pad Prizm v. 6. Groups were compared using the Mann-Whitney U-test for independent small datasets. The normality of distribution was checked using the D'Agostino-Pearson test. Differences were considered as statistically significant at $p \leq 0.05$.

RESULTS

The Effect of PAE on the Expression Level of TLR Genes

On day 8 after birth the levels of TLR3 and TLR4 mRNAs demonstrated the 1.46- and 1.75-fold increase in the forebrain cortex of animals of the group PAE+saline. The level of TLR7 mRNA did not change significantly (Fig. 1). Analysis of the mRNA content of adapter proteins (MYD88, TICAM) (Fig. 2) and transcription factors (NF-κB, IRF1, IRF3, IRF7) (Fig. 3), involved in TLR-mediated signaling, revealed a 1.73-fold increase in the TICAM mRNA content (Fig. 2). Analysis of the mRNA content of pro-inflammatory (IL-1β, TNF-α, IL-6, IFN-γ, CCL2) (Fig. 4) and anti-inflammatory cytokines (TGF-β, IL-13, IL-10, IL-11, IL-4) (Fig. 5) showed that on day 8 the level of the following mRNAs increased in the forebrain cortex of animals: IL-1β (by 1.38 times), TNF-α (by 1.54 times), IFN-γ (by 1.94 times), CCL2 (by 1.29 times), IL-13 (by 1.88 times), IL-10 (by 3.64 times), IL-11 (by2.24 times), IL-4 (by 1.71 times). An increased level of HMGB1 mRNA was also noted (by 1.54 times) (Fig. 6). This protein is known to mediate activation of the TLR4 signaling, which leads to the expression and secretion of pro-inflammatory cytokines.

Table 1. Sequences of primers used in this study

Gene	Primers	
	Forward (5′-3′)	Reversed (5′-3′)
Tlr3	AACTGGAGAACCTCCAAGA	CACCCTGGAGAAAACTCTTT
Tlr4	ACTCTGATCATGGCATTGTT	GTCTCAATTTCACACCTGGA
Tlr7	TGAAAATGGTATTTCCAATGTG	TAAGGGTAAGGTTGGTGGTA
Hmgb1	CTCTGATGCAGCTTATACGA	AAAAGACTAGCTTCCCCTTG
Myd88	TCATTGAGAAAAGGTGTCGT	AGTGCAGATAGTGATGAAC
Ticam	GCTCAGCTAGATGATGTGAT	TGACAGTGCAGACCTGG
NfκB	ATACTGCTTTGACTCACTCC	AGGTATGGGCCATCTGTT
Irf1	CGGAAGTTACCTTCTAGCTC	CGGAAGTTACCTTCTAGCTC
Irf3	AATTCCTCCCCTGGCTC	CATGGGATCCTGAACTTTGT
Irf7	TTGGTTACACTATCTGTGGC	CTACTGACCTCACCCAAGA
Il1β	TGTCTGACCCATGTGAGCTG	TTTGGGATCCACACTCTCCAG
Ccl2	AAGATGATCCCAATGAGTCG	TGGTGACAAATACTACAGCTT
Il10	CTGCAGGACTTTAAGGGTTA	CCTTTGTCTTGGAGCTTATT
Il6	ACTTCACAAGTCGGAGGCTT	AATTGCCATTGCACAACTCTTTTC
Tnfα	CACGTCGTAGCAAACCAC	TATGAAATGGCAAATCGGCT
Tgfβ	GGACTACTACGCCAAAGAAG	GGTTTTGTCATAGATTGCGTT
Il13	TGTAACCAAAAGGCCTCGGA	TGGCCATAGCGGAAAAGTTG
Il4	CGGTGAACTGAGGAAACT	TCAGTGTTGTGAGCGTGG
Ifnγ	AGCCTAGAAAGTCTGAAGAAC	ATTTTCGTGTTACCGTCCTTT
Il11	GGGACATGAACTGTGTTTGT	GGTAGGTAGGGAGTCCAGAT
Gapdh	GCCAGCCTCGTCTCATA	GTGGGTAGAGTCATACTGGA

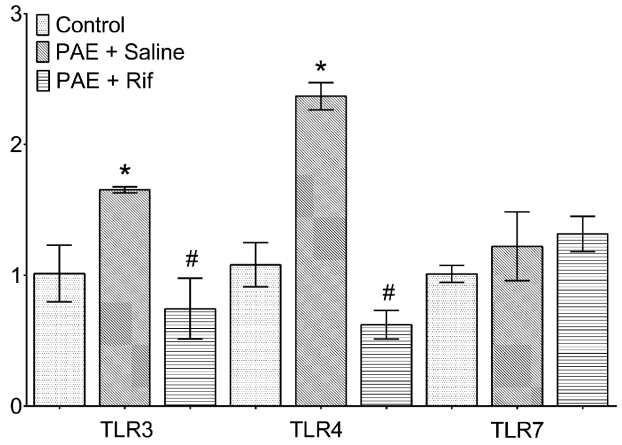

Figure 1. The content of TLR mRNA in the forebrain cortex of rats on day 8 of their neonatal development (arbitrary units, mean values ± SEM; * – $p \leq 0.05$ versus control, # – $p \leq 0.05$ versus the PAE group).

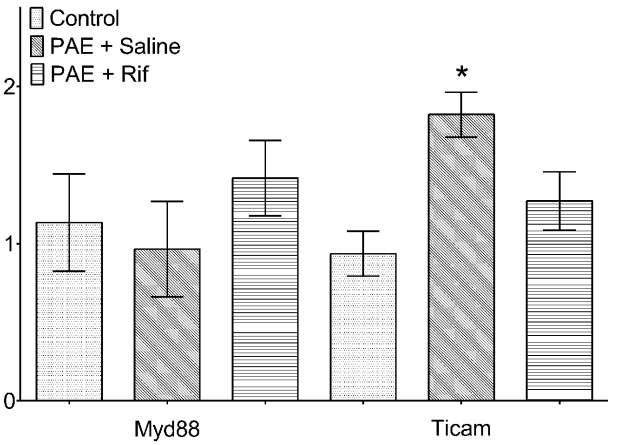

Figure 2. The content of mRNA of adapter proteins in the forebrain cortex of rats on day 8 of their neonatal development (arbitrary units, mean values ± SEM; * – $p \leq 0.05$ versus control).

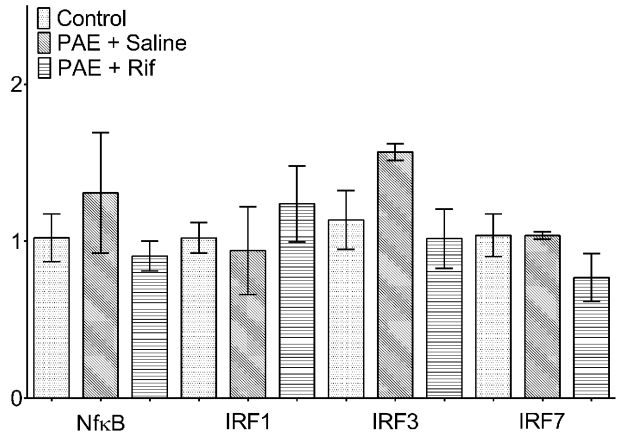

Figure 3. The content of mRNA of transcription factors in the forebrain cortex of rats on day 8 of their neonatal development (arbitrary units, mean values ± SEM; * – $p \leq 0.05$ versus control, # – $p \leq 0.05$ versus the PAE group).

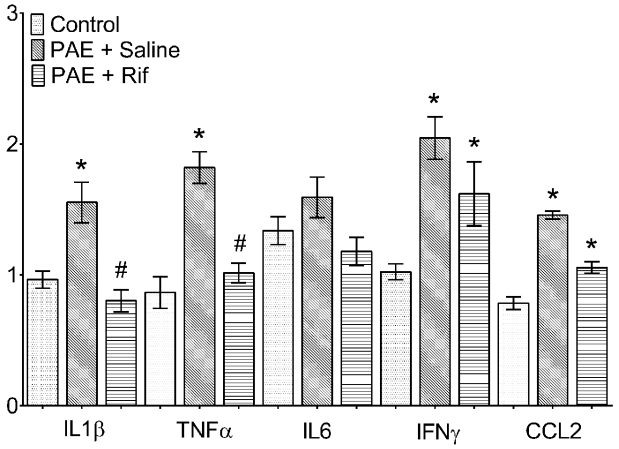

Figure 4. The content of mRNA of pro-inflammatory cytokines in the forebrain cortex of rats on day 8 of their neonatal development (arbitrary units, mean values ± SEM; * – $p \leq 0.05$ versus control, # – $p \leq 0.05$ versus the PAE group).

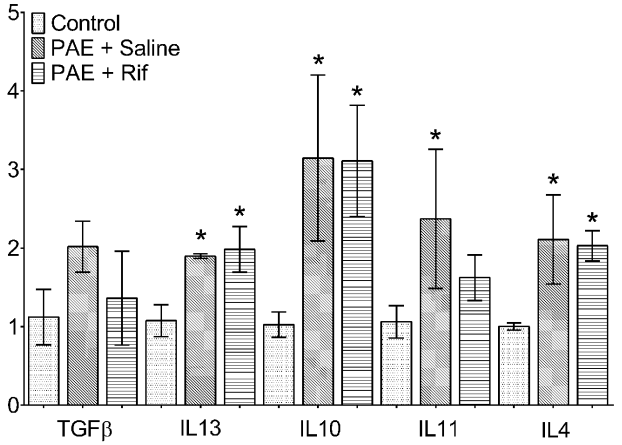

Figure 5. The content of mRNA of anti-inflammatory cytokines in the forebrain cortex of rats on day 8 of their neonatal development (arbitrary units, mean values ± SEM; * – $p \leq 0.05$ versus control).

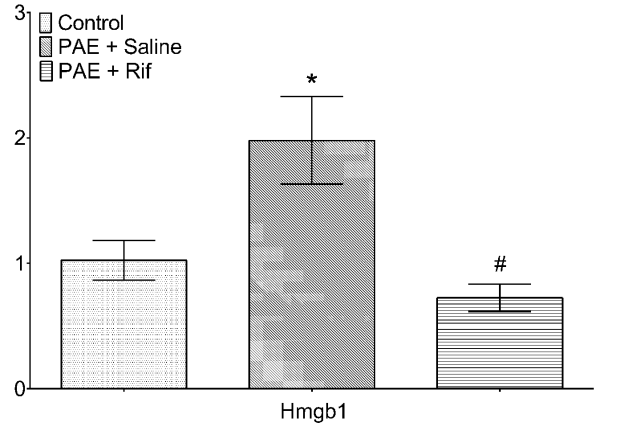

Figure 6. The content of HMGB1 mRNA in the forebrain cortex of rats on day 8 of their neonatal development (arbitrary units, mean values ± SEM; * – $p \leq 0.05$ versus control, # – $p \leq 0.05$ versus the PAE group).

The Influence of Rif Administration on the Expression Level of Genes of the TLR-Signaling Pathways of Cytokines in the Forebrain Cortex of Animals Subjected to PAE

Administration of Rif (50 mg/kg ip) to animals with PAE from day 1 to day 7 of neonatal development caused a decrease in the mRNA level of TLR3 (by 2.23 times) and TLR4 (by 3.02 times) to the control level (Fig. 1). No statistically significant differences were found between the group PAE+Rif and control. In the group PAE+Rif, no statistically significant changes in the mRNA of the studied adapter proteins (Fig. 2) and transcription factors (Fig. 3) were detected versus control. However, administration of Rif normalized the mRNA level of the proinflammatory cytokines IL-1β ($p \leq 0.05$) and TNF-α ($p \leq 0.05$) (Fig. 4), as well as the content of HMGB1 mRNA ($p \leq 0.05$) to the control level (Fig. 6).

DISCUSSION

Alcohol intake causes various changes in the TLR signaling system in the brain structures [15–20]. Using TLR agonists and antagonists, as well as genetic manipulations, several groups of researchers confirmed the fact that changes in the activity of the TLR signaling system mediated the development of neuroinflammation in the nervous tissue [18, 19, 21–27].

Good evidence exists that all TLR subtypes are expressed in the brain, starting from the earliest stages of ontogenesis [28, 29]. Changes in the TLR-signaling system of the brain were detected in offspring with some prenatal pathologies [30–33]. We have hypothesized that PAE may cause long-term changes in the TLR signaling system in the developing brain.

The PAE modeling performed in this study revealed increased levels of forebrain cortex TLR3 and TLR4 mRNAs on day 8 of postnatal development of rats subjected to PAE. We did not detect any changes in the content of TLR7 mRNA in the forebrain of the animals subjected to PAE. It is possible that the TLR7 system is more resistant to PAE in the cortex, or changes in the expression of the *Tlr7* gene are less long-lasting.

Administration of Rif in our experiment reduced the level of TLR3 and TLR4 mRNAs in the group of animals exposed PAE to the control level. We have chosen this compound based on the known information about Rif effectiveness in different models of neuroinflammation, including the ability of Rif to reduce the level of pro-inflammatory cytokines, the content of β-amyloid in the model of Alzheimer's disease, and the level of α-synuclein in the model of Parkinson's disease [33–36].

Our study has shown that in the group of animals with PAE, the level of HMGB1 mRNA increased in the forebrain cortex on day 8 of postnatal development. In the extracellular space, the HMGB1 protein is able to interact with TLR2, TLR4, TLR5, and TLR7, thus causing activation of intracellular signaling pathways mediated effects of these receptors [37, 38]. Administration of Rif decreased the level of HMGB1 mRNA to control values. Determination of the mRNA level of TLR-signaling components showed an increased content of TICAM and IRF3 mRNAs in the forebrain cortex on day 8 of postnatal development of animals subjected to PAE. At the same time, in the group of animals PAE+Rif, the level of IRF3 mRNA corresponded to the control values.

In the group of animals PAE+saline the mRNA levels of IL-1β, TNF-α, IFN-γ, and CCL2 increased. This suggests long-term changes in gene expression of pro-inflammatory cytokines in the developing brain of animals subjected to PAE. However, the molecular causes that initiate these events, as well as the consequences of such changes (including changes in the level of proteins encoded by these genes) are still to be investigated. Further studies are clearly needed to investigate whether changes in the mRNA level of anti-inflammatory cytokine are accompanied by changes in the corresponding protein products.

Thus, on day 8 of postnatal development of animals subjected to PAE the increase in the activity of neuroinflammation pathways probably initiates activation of genes involved in the anti-inflammatory/neuroprotective pathways. It is likely that both the increased expression level of proinflammatory genes and the increased expression level of genes related to the functioning of the anti-inflammatory/neuroprotective mechanisms may have adverse effects on the normal course of neurogenesis in the offspring, which may cause formation of cognitive and other disorders associated with the FASD formation in the future.

Administration of Rif led to a significant decrease in the mRNA level of the key pro-inflammatory cytokines TNF-α and IL-1β but had insignificant effects on the mRNA levels of other cytokines analyzed in this study. This may be due to the fact that the dosage of Rif was insufficient to eliminate all the changes in the expression of pro-inflammatory cytokine genes. In addition, it can be assumed that other molecular intracellular mechanisms that have not been investigated yet contribute to the dysregulation of the expression level of cytokine genes associated with PAE.

CONCLUSIONS

Our study provided new data on the expression state of genes of the TLR signaling system on day 8 of postnatal development in the forebrain cortex

of animals subjected to PAE. The results of the study showed the ability of Rif to correct the observed long-term pathological changes in the TLR system. A decrease in elevated levels of TLR3, TLR4, HMGB1, IL-1β, and TNF-α mRNA in the forebrain cortex of animals subjected to PAE was found.

FUNDING

The work was carried out within the framework of the State Task of the Ministry of Education and Science of Russia (2022-2025) "Search for molecular targets for pharmacological effects in addictive and neuroendocrine disorders and the creation of new pharmacologically active substances acting on CNS receptors", code FGWG-2022-0004.

COMPLIANCE WITH ETHICAL STANDARDS

The methods used in the work were approved by the Ethical Committee for the Care and Use of Animals of the Institute of Experimental Medicine (protocol No. 21/5 dated February 26, 2015).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Received: 10. 04. 2023.
Revised: 20. 05. 2023.
Accepted: 08. 06. 2023.

ВЛИЯНИЕ РИФАМПИЦИНА НА ЭКСПРЕССИЮ ГЕНОВ СИСТЕМЫ TOLL-ПОДОБНЫХ РЕЦЕПТОРОВ В КОРЕ ПЕРЕДНЕГО ОТДЕЛА МОЗГА КРЫСЯТ С ПРЕНАТАЛЬНЫМ ВОЗДЕЙСТВИЕМ АЛКОГОЛЯ

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Этанол служит причиной длительных изменений в системе toll-подобных рецепторов (TLR), способствуя активации путей нейровоспаления. Употребление алкоголя во время беременности вызывает нейровоспалительный процесс у плода, что может приводить к развитию симптомов фетального алкогольного спектра нарушений (ФАСН). В нашем исследовании показано, что пренатальное воздействие алкоголя вызывает долгосрочные изменения в экспрессии генов системы TLR-сигнализации (*Tlr3*, *Tlr4*, *Ticam*, *Hmgb1*, генов цитокинов) в коре переднего отдела мозга крысят. Применение рифампицина (Rif), способного снижать уровень провоспалительных медиаторов при различных патологических состояниях нервной системы, нормализовало изменённый уровень экспрессии исследуемых генов TLR-сигнализации. Это свидетельствует о том, что Rif может предотвращать развитие стойких нейровоспалительных явлений в коре переднего отдела мозга крысят, вызванных дисрегуляцией в системе TLR.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: мозг; ПВА; алкоголь; toll-подобные рецепторы; нейровоспаление; рифампицин

Финансирование. Работа выполнена в рамках государственного задания Минобрнауки России (2022-2025 гг.) “Поиск молекулярных мишеней для фармакологического воздействия при аддиктивных и нейроэндокринных нарушениях и создание новых фармакологически активных веществ, действующих на рецепторы ЦНС”, шифр FGWG-2022-0004.

Поступила в редакцию: 10.04.2023; после доработки: 20.05.2023; принята к печати: 08.06.2023.